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Original article

Development of HPTLC Fingerprinting and Phytochemical Study of a Polyherbal Unani Formulation

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ABSTRACT

Plants produce a wide range of active principles, making them a rich source of different types of medicines. Without any knowledge of the phytoconstituents or active principles the medicinal plants itself or in the form of polyherbal formulations, were used by all society of human being from ancient times for prevention and cure of disease, but most of the traditional formulations including the formulation of Ayurveda and Unani have not been scientifically validated in order to establish the pharmacopoeial standards to improve the efficacy. Globally, the people become conscious that uses of synthetic drugs for a long period is not safe; the trend of medical society at large is looking at alternatives from natural, safe sources to combat diseases. Due to this comprehension, it has been increased the demand for plant-derived medicine, and on the other side, it is extremely important to standardize the polyherbal formulations and validate them scientifically to improve their safety and efficacy.

The polyherbal Unani formulation Safuf-e-Muallif is widely used and recommended in Unani system of medicine (USM) as a spermatogenic agent, semen thickening agent, etc. to treat sexual disorders viz. premature ejaculation, nocturnal emission, etc. The study mainly deals with phytochemical screening for the detection of nature of phytoconstituents and analytical technique like High-performance thin-layer chromatography (HPTLC) for developing fingerprint of Safuf-e-Muallif revealing specific identities of the drug. The phytochemical screening and HPTLC fingerprint profile for SM reported here may be used as a reference standard for quality control purpose in future.

Keywords: Standardization, Phytochemical analysis, HPTLC, Unani Medicine.

INTRODUCTION

Medicinal plants are one of the best resources from nature and their utilization for the benefits of humankind. According to the world health organization, approximately 80% of the world population still depends on traditional medicine, as an important source of medicine (Ramanjaneyulu & Bhargavi, 2011). India is a land where various traditional systems of medicine are existed together and based on natural sources. Unani system of medicine (USM) is also a part of the traditional system of medicine and mainly centered naturally occurring drugs, mostly plant sources. The drugs obtain from animal and mineral origin is also being used (Mohd, Sifi, Jahan & Baig, 2017). In USM, plants origin drugs are extensively used both as single and compound formulations as a rich source of therapeutic agents for the prevention of diseases and ailments. Recent years there has been a move in universal trend from synthetic to herbal medicine (Anonymous, 2016). Globally the revival of interest in herbal medicines has led to an increase in their demand leading to a decline in their quality, primarily due to a lack of adequate regulations affecting drugs (Sharma et al., 2008). WHO has also emphasised the need to ensure quality control of medicinal plant products by using sophisticated modern analytical techniques. Curative efficacies of compound herbal medicine are reliant on the quality and the quantity of the active constituents of single drugs as they have specific pharmacological actions. Though plants vary in there curing properties for a reason that of their bioactive constituents. The phytoconstituents are also known as plant secondary metabolites which are available in less quantity in plants and chemically it is of various types like alkaloids, glycosides, steroids, tannins and saponin. Hence, it is very important that to identify the polyherbal Unani formulation for its active constituents by using the modern methods and High-Performance Thin Layer Chromatography (HPTLC) finger-printing.

Safuf-e-Muallif (SM) is a polyherbal formulation in powder form used in Unani System of Medicine for treatment of sexual disorders since ancient time, and the formulation is mentioned in Qarabadin-e-majidi, a classical Unani Pharmacopoeia (Anonymous, 1986). The study formulation consists of following ingredients Tal Makhana (*Asteracantha longifolia*

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Nees.), Salab Miṣri (*Orchis latiflolia* L.), Singhara Khushk (*Trapa bispinosa* Roxb.), Gond Kikar (*Acacia arabica* Willd.), Mazu Sabz (*Quercus infectoria* Oliv.), Mastagi Rumi (*Pistacia lentiscus* L.), Nishashtah Gandum-Starch (*Triticum aestivum* L), Sugar (*Saccharum officinarum* L.) used and recommended in Unani system of medicine as a spermatogenic agent, semen thickening agent, etc. to treat sexual disorders viz. premature ejaculation, nocturnal emission. This formulation was not studied for its phytoconstituents nature and also not develops HPTLC fingerprinting profile as per the literature. Therefore SM was subjected to preliminary phytoconstituents analysis and high-performance thin-layer chromatographic studies. The present study describes the salient features of phytoconstituents presence and HPTLC profile of SM.

MATERIALS AND METHODS

Plant Ingredients of the formulation SM were procured from the GMP certified pharmacy, identified and authenticated by the Pharmacognosist at National Research Institute of Unani Medicine for Skin Disorders, Hyderabad, India. All the solvents used in the study area were of HPLC grade. DESAGA Sarstedt Gruppe system HPTLC instrument (Germany) was used along with automatic TLC applicator and UV visible cabinet as an imaging system, proquant 1.6 version software. Twin-trough chamber 20 X 10 cm was used for development, stationary phase as a Pre-coated silica gel 60 F254 Thin Aluminium plates (Merck, KgaA, Germany).

1. Preparation of formulation

SM was prepared as per the prescribed procedures mentioned in the Qarabadin-e-majidi, a classical Unani Pharmacopoeia (Anonymous, 1986); National Formulary of Unani Medicine (Anonymous, 2006); Unani pharmacopoeia of India (Anonymous, 2007).

2. Preparation of extracts for preliminary phytochemical study

The preliminary phytochemical study was carried out using different solvent extracts of the SM, i.e., alcoholic, aqueous and chloroform extracts for determination of different class of compounds present. The alcoholic extract was prepared by taking five grams of SM powder in a conical flask, and 100 ml ethanol was added and placed over orbital for 6-8 hours for shaking at a speed of 120-130 rpm. The flask was removed, and the content of the flask are filtered and evaporated and concentrate on getting the dry extract for the constant weight. Similarly, in the same way, prepare the aqueous and chloroform extract using the respective solvents (Yadav & Agarwal, 2011; Rasheed, Nagaiah, & Waheed, 2012; Rasheed et al., 2012).

3. Qualitative phytochemical screening

3.1 Detection of alkaloids: The small portions of the extract of SM are stirred separately with a few drops of dilute HCl and filtered and then subjected to test for the presence of alkaloids.1. Dragendroff's test: To 2 ml of extract was treated with Dragendroff's reagent. Formation of a reddish-brown precipitate indicates the presence of alkaloids. 2. Mayer's test: To 2 ml of extract was treated with Mayer's reagent (potassium mercuric iodide solution). Formation of a cream-yellow precipitate indicates the presence of alkaloids. 3. Wagner's test: To 2 ml of extract was treated with Wagner's reagent (iodine solution). Formation of a reddish-brown precipitate indicates the presence of alkaloids. 4. Hager's test: To 2 ml of extract was treated with Hager's test reagent (a saturated solution of picric acid in cold water). Formation of a yellow precipitate indicates the presence of alkaloids.

3.2 Test for Carbohydrates: 1. Molisch's test: A small quantity of extract was dissolved in 1 ml of distilled water and filtered it. After that, the filtrate was treated with two drops of alcoholic α-naphthol solution in a test tube, and 2 ml of concentrated sulphuric acid was added carefully along the sides of the test tube wall. Formation of violet colour ring at the junction indicates the presence of carbohydrates. 2. Fehling's test: To 2 ml of aqueous extract of the drug was taken in a test tube adds an equal part of with Fehling's solution-A & Fehling's solution–B, boiled gently and observed. A precipitate of the brick red colour formed which indicate the presence of reducing sugars. 3. Benedict's test: Alcoholic and aqueous extract of SM was treated with Benedict's reagent and heated on the water bath. Formation of an orange-red precipitate indicates the presence of reducing sugars.

3.3 Test for Glycosides: 1. Chrysarobin test: Two gram of powder of SM was dissolved in 3 ml of Conc. H_2SO_4, a deep red solution is produced, which indicates the presence of glycosides. 2. Anthraquinone glycoside test: The powdered formulation SM was extracted with ether or any immiscible water solvents. The filtered ethereal extract is made alkaline either with NaOH or NH_3. The aqueous layer shows a pink colour, turns red to violet colour indicates the presence of anthraquinone glycoside.

3.4 Test for phenolic compounds: 1. Ferric chloride solution: To 2 ml of alcoholic and aqueous extracts of the SM was taken in the test tube, then added two ml of ferric chloride solution and observed for the formation of green and blue colour. It shows the presence of the phenolic compound. 2. Liebermann's nitroso test: Take 2 ml of alcoholic and aqueous extracts of the SM then adds few ml of Liebermann's nitroso reagent ($NaNO_2$ and Conc. H_2SO_4) and observed. It provides a deep blue colour which changes to red on dilution with water.

3.5 Test for protein and amino acids: 1. Biuret test: To the extract, two drops of biuret reagent were added and observed for the formation of red colour. 2. Millon's test: To 2 ml of alcoholic and aqueous extract of SM was taken in a test tube and added to five-six drops of Millon's reagent (Solution of mercury nitrate and nitrous acid) and observed for the formation of a red precipitate which indicates the presence of amino acids.

3.6 Test for Saponin: Foam test: To the alcoholic extract of SM few drops of sodium bicarbonate were added, shaken well and observed for the formation of honeycomb-like frothing, which indicates the presence of saponin.

3.7 Test for steroids and triterpenes: 1. Libermann-Burchard's test: Few ml of alcoholic and aqueous extract of SM was taken in a separate test tube. Each extract was treated with few drops of acetic anhydride; it was gently heated and cooled. Few drops of concentrated sulphuric acid were added through sides of the test tube. It forms a reddish colour ring at the interface which indicates the presence of steroids and triterpenes. 2. Salkowski's Test: The alcoholic extract was treated with chloroform and filtered. The filtrates were treated with a few drops of concentrated sulphuric acid, shaken and allowed to stand for appearance of yellow ring at the junction, which turns red after one minute, indicates the presence of steroids and triterpenes. (Yadav & Agarwal, 2011; Rasheed, Nagaiah, & Waheed, 2012; Rasheed et al., 2012)

4. High-Performance Thin Layer Chromatography (HPTLC) analysis

Thin-layer chromatography and HPTLC is one of the important separation chromatographic techniques used for detecting the adulteration for assessing the quality of the drugs through fingerprint profile of the drug. If the drug is adulterated there

might be the appearance of the other compounds, in turn may increase the no of spots. On the other hand, the exhausted or deteriorated drugs may lose the component, and the number of spots appeared might be less.

High-performance thin-layer chromatography (HPTLC) is a popular method for quality control of herbal products and the analysis of herbal medicines. It is widely used for separation, qualitative and quantitative estimation of marker compounds present in herbal drugs. HPTLC fingerprint profile is suitable for standardization of components followed by determination of specific bio-active phytoconstituents from plant materials. The HPTLC fingerprint for the formulation was developed. Major advantage of HPTLC is its ability to analyse several samples simultaneously using a small quantity of sample (Khandelwal, 2002; Anonymous, 2011; Qureshi, 2018).

4.1 Preparation of alcoholic extract of the SM for HPTLC analysis

Five gram of powdered sample is taken and reflux with 200 ml of alcohol using a soxhlet apparatus on a water bath for 30 minutes. Filter the extract and concentrate to 5 ml then the sample extract obtained so far is used for further analysis.

4.2 HPTLC method conditions

The sample extract was spotted on pre-coated aluminium sheets of silica gel 60 F254 (Merck) with the help of automatic TLC applicator system of the DESAGA Sarstedt Gruppe. After trying with various solvent systems with variable volume ratios, the suitable solvent system as stated in its selected proportional ratio and developed in the twin through chamber of TLC to the 80mm height of the plate to separate the components on the polar phase of silica gel and that of the mobile phase of the solvent system.

S.No.		**Method conditions**
1	Made/ Make of Instrument	Desaga Sarstedt Gruppe (Germany),
2	Development Chamber	20 X10 cm, Twin-trough chamber
3	Stationary phase	Pre-coated silica gel 60 F254 Aluminium plates (Merck, KgaA, Germany)
4	Plate thickness	0.2 mm
5	Plate size	200 x 100 mm
6	Distance from starting	20 mm
7	Distance from bottom	10 mm
8	Volume applied	5 µl
9	Band length	10 mm
10	Distance between tracks	20 mm
11	Development distance	80 mm

4.3 Development of HPTLC technique

After developing, TLC plate was air-dried and detected with the suitable detection system like UV Cabinet system for detection of spots at 366nm, 254nm and also under iodine vapours and after derivatising with anisaldehyde sulfuric acid reagent as shown in the figure 1. Further, it was scanned with the Densitometer CD60 of DESAGA Sarstedt Gruppe system under the UV range of 366nm, 256nm, under exposure to iodine vapours at 580nm and after derivatisation with anisaldehyde sulfuric acid reagent at 580nm. The typical densitograms obtained upon scanning under densitometer under the specific conditions for the above detection system were shown, which peaks appeared for the corresponding spots being detected in the densitometer. The peak areas in the densitogram correspond to the concentration of the component in the sample. The suitable separation of the components was developed for the important formulation, and the R_f values were recorded.

Table 1. Analysis of phytochemical constituent of the SM

Phytochemical constituent	**Observation**		
	Ethanolic extract	**Aqueous extract**	**Chloroform extract**
Alkaloid	-	-	-
Carbohydrates	+	+++	-
Fixed oil	+	++	-
Glycosides	++	++	+
Phenols	+	++	+
Resins	+	+	+
Saponin	-	++	-
Protein	-	++	-
Starch	+	++	-
Steroids	+	-	-
Tannins	-	+	+

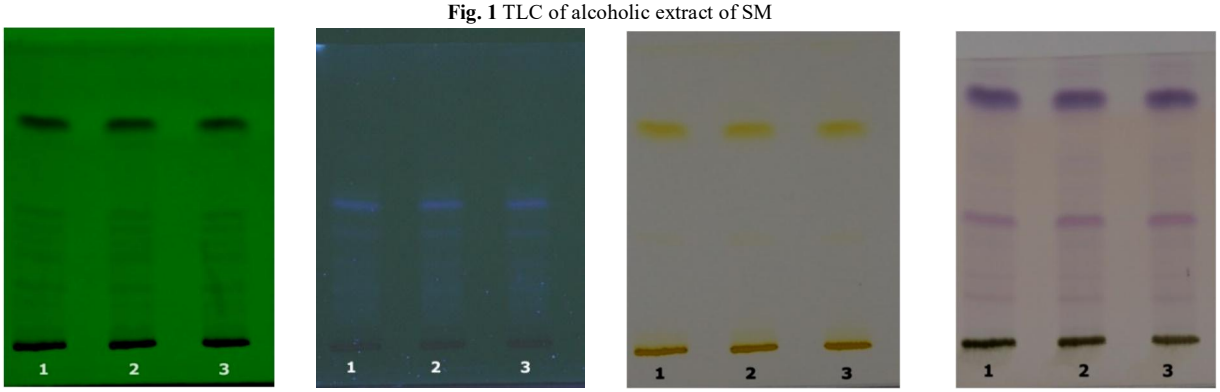
RESULT AND DISCUSSION

Phytochemical screening of SM- Phytochemical screening was carried out through qualitative test in different solvent extracts such as alcoholic, aqueous and chloroform extracts of the Safuf-e Muallif and the nature of Phyto-constituents found in the formulation were recorded as shown in the table 1. On qualitative test performed to detect various class of compound such as alkaloids, carbohydrates, fixed oil, glycosides, phenols, resin, saponins, protein, starch, steroids, tannins etc. The results indicated the presence of carbohydrate, glycosides; phenols, resins, saponins, proteins, starch, steroids, tannins and fixed oil are present. Among above nature of constituent's carbohydrate, glycosides, fixed oil, starch were found to be strongly positive showing its major presence whereas the very weak or negative presence of alkaloid and tannins were found to be present.

HPTLC analysis of an alcoholic extract of study formulation
The alcoholic extract of SM 10 µl was spotted on silica gel "G" plate using applicator and developed in twin through a chamber with Toluene: Ethyl Acetate: Methanol (7:2:1, v/v/v) as mobile phase. The air-dried TLC plate shows four major spots under UV 366nm at R_f values 0.18 (blue), 0.25 (blue), 0.37 (blue), 0.48 (blue) ; and under UV 254nm shows five spots at R_f values 0.17, 0.28, 0.37, 0.42 and 0.75 (all black); and under iodine vapours shows two spots at R_f values 0.35, 0.72 (both are brown) under visible region after derivatizing with anisaldehyde sulphuric acid (ASA) reagent and heating at 105 ^{0}C shows four spots at R_f values 0.14, 0.21, 0.37 and 0.78 (all purple) as shown in table 2-5 and Densitogram representation shown in figure 2-5 respectively.

In our study, the TLC studies of alcoholic extract of SM was performed for the separation of different compounds present in the solvent extract, and R_f values of various spots appeared in the TLC plate were calculated respectively. The TLC of alcoholic extract of SM with mobile phase solvent system as toluene: ethyl acetate: methanol (7:2:1, v/v/v) was developed and detected in various detection system such as UV 366nm, 254nm, exposure to iodine vapours and after derivatisation with anisaldehyde sulphuric acid reagent is studied. The R_f values corresponding to each spots was observed and recorded as four major spots appeared under detection of UV 366nm at R_f values 0.18 (blue), 0.25 (blue), 0.37 (blue), 0.48 (blue) ; and under UV 254nm shows five spots at R_f values 0.17, 0.28, 0.37, 0.42 and 0.75 (all black); and under iodine vapours shows two spots at R_f values 0.35, 0.72 (both brown) under visible region after derivatizing with anisaldehyde sulphuric acid and heating at 105 ^{0}C shows four spots at R_f values 0.14, 0.21, 0.37 and 0.78 (all purple).

Fig. 1 TLC of alcoholic extract of SM



At UV 254nm | At UV 366nm | under iodine vapours | after derivatization with ASA reagent

Table 2. Peak list of alcoholic extract of SM at UV 366nm

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	9.6	1363.78	96.25	473.13	0.01
2	28.4	3.92	0.28	3.44	0.27
3	38.2	10.04	0.71	7.96	0.41
4	43.9	36.02	2.54	14.40	0.48
5	75.3	3.14	0.22	3.61	0.92

Fig.2. Densitogram of alcoholic extract of SM at UV 366 nm

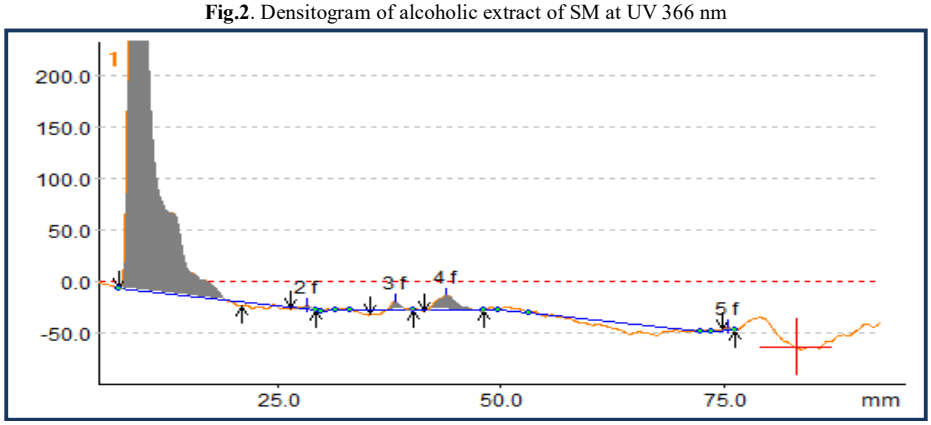


Table 4. Peak list of alcoholic extract of SM upon exposure to iodine vapours

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	9.5	891.93	80.51	483.15	0.01
2	62.7	212.70	19.20	71.18	0.71
3	82.1	3.27	0.30	4.51	0.96

Fig. 4 Densitogram of alcoholic extract of SM upon exposure to iodine vapours

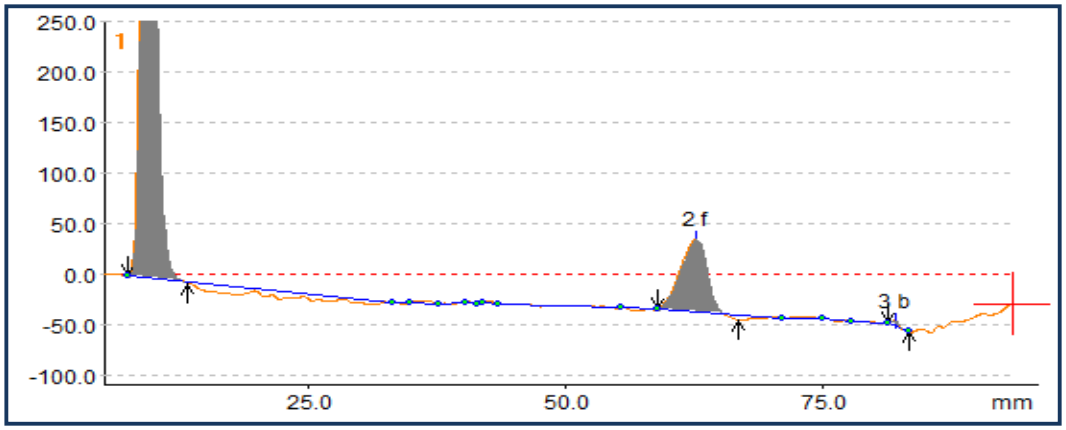
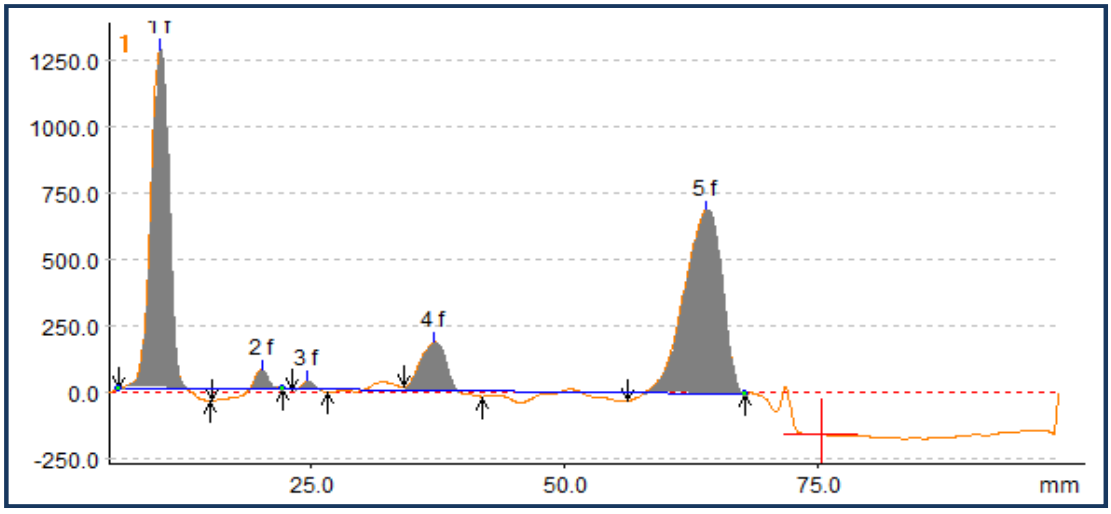


Table 5. Peak list of alcoholic extract of SM upon derivatised with anisaldehyde sulphuric acid at 580 nm

Peak no	Y-Pos	Area	Area %	Height	R_f value
1	10.1	2567.07	41.67	1259.04	0.02
2	20.1	104.61	1.70	71.64	0.15
3	24.7	44.22	0.72	32.54	0.22
4	37.2	520.79	8.45	180.74	0.39
5	64.1	2924.03	47.46	683.05	0.77

Fig. 5 Densitogram of alcoholic extract of SM upon derivatised with anisaldehyde sulphuric acid (ASA) reagent



TLC helps in determining the number of constituents present in the extract of formulation and predict the nature of polarity of the compounds. Further, the HPTLC analysis was carried to obtain the corresponding densitogram in which different peaks are observed for the spots appeared in the TLC plate under the various detection systems. The HPTLC technique is very much efficient and important to detect the number of components present in the extract using the peaks recorded in the densitogram.

CONCLUSION

The polyherbal Unani formulation SM, upon phytochemical screening and development of HPTLC fingerprinting by using modern analytical tool helps in establishing the standard parameters and quality standards. Modern HPTLC technique of chromatography was employed in the standardization which helps to separate the compounds those may be isolated for further studies to generate markers for the formulation. Consequently, the drug was brought up in determining and ascertaining its quality standards. The study also helps in quality assurance of SM various batches and its reproducibility with consistency. Moreover, the developed quality standard may also helpful in the production of efficacious Unani formulations in future.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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